Toxic effects of mazut on early developmental stages of blenny *Parablennius sanguinolentus*

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Abstract

Coastal marine waters are known as spawning sites of fish and invertebrates, which are highly sensitive to crude oil and its derivates in their early developmental stages. The aim of the present study was to investigate the effects of mazut at concentrations of 0.00001, 0.02, 0.1, and 0.2 ml/l on antioxidant enzyme activities in developmental embryos (stage V) of the Black Sea blenny *Parablennius sanguinolentus*. The biomarkers of oxidative stress — namely superoxide dismutase (SOD), catalase (CAT), peroxidase (PER) and glutathione reductase (GR) — were investigated. This revealed a non-uniform impact of mazut concentration on fish embryos. The correlations between enzyme activities and mazut concentrations were non-linear, and tested antioxidants fluctuated independently from one another. The obtained results can be applied to the development of oil toxicity tests for assessment of water quality and in conservation biology.

Keywords: Black Sea, mazut, fish eggs, blenny *Parablennius sanguinolentus*, antioxidant enzymes, oil pollution, biomarkers, oxidative stress, monitoring, risk assessment

Introduction

Contamination of marine waters by petroleum hydrocarbons (PHs) such as diesel, gasoline, mazut and other typical PHs is a global problem for aquatic ecosystems, since they are harmful for aquatic life because of their highly toxic, mutagenic, and carcinogenic effects. PHs usually disrupt the cell membrane, change its fluidity, integrity, and organism function. Non-bioavailable and/or hydrophobic PHs become bioavailable to several benthic organisms (e.g., invertebrates, adult fish and fish eggs) as they get adsorbed onto particulates and sediments (Kuppusamy et al., 2020). The marine environment acts as a recipient for different kinds of contaminants, including heavy metals, organic substances, nanoparticles, plastics, pharmaceuticals and oil. The negative effects of oil contamination in marine ecosystems are well-known for many seas and oceans, including the Black Sea (Cokacar, 2008; Oguz 2017; Sardi et al. 2017; Carpenter and Kostianoy, 2019).

Oil pollution remains an important problem for marine ecosystems, because oil spills and other accidents are frequent events in the marine environment. Therefore, development of corresponding tests for the evaluation of their consequences for aquatic biota is very important (Embry et al., 2010; Pereira et al., 2018). For the development of oil toxicity tests, various test organisms are used, including early developmental stages of marine fish and invertebrates (Franco et al., 2018). Fish embryo testing in ecotoxicological studies has been largely utilized as a good tool for toxicity assays. Zebrafish (*Danio rerio*) embryos were used for evaluation of the toxicity of unrefined crude and residual fuel oils, along with the effects of sunlight as an additional stressor (Martin et al., 2014). Because

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early life stages of marine fish are very sensitive to oil pollution, studies have been performed using the different life stages of Japanese medaka (*Oryzias latipes*), fathead minnow (*Pimephales promelas*), goldfish (*Carassius auratus*), rainbow trout (*Oncorhynchus mykiss*), etc. Despite this, data concerning the toxicity of pollutants in fish eggs and larvae remain scarce (Samuelsen et al., 2019; Blahova et al., 2020).

The risk of oil contamination is accompanied by extreme consequences for marine ecosystems, with physical, chemical and biological damage of the water, sediments and biota. Petroleum contamination is chronic in the Black Sea. Oil is distributed in the surface water and accumulated in the bottom sediments and organisms (Matishov et al., 2016).

Early life stages of fish and invertebrates are very sensitive to pollution by oil and its derivates. Researchers have reported many negative effects of oil contamination on fish eggs and larvae (Rudneva, 2014; Hansen et al., 2018; Mu et al., 2018; Pereira et al., 2018). Oil induces a series of abnormalities during fish embryonic development, increases embryo mortality at various stages, changes morphogenesis and organ formation, and modifies the responses of many metabolic processes (Martinez-Gomez et al., 2007; Paula et al., 2011; de Andrade et al., 2018; Mu et al., 2018). In this context, antioxidant biomarkers and their response to crude oil and its derivates in fish and invertebrates in field and experimental conditions are good tools for the assessment of toxic effects of petroleum on early fish life stages and for biomonitoring. However, information about biomarker response in early life stages of aquatic organisms is scarce, despite many of them being used in national and international monitoring programs for evaluation of ecosystem water quality, including the consequences of oil accidents and spills.

The blenny *Parablennius sanguinolentus* is a benthic settled euryphagous fish with a diet of small invertebrates, namely mollusks, crustaceans and polychaets. Before reproduction the male constructs a burrow under stones and various benthic substrates, which is then used for shelter and spawning. The male forces females into the burrow; several females can participate in the spawning process. After fertilizing the eggs, the male guards them from predators and keeps them free from detritus. During this period the male is very aggressive against predators and other animals which approach the burrow. It fans the eggs, using the vibration of dorsal and caudal fins to provide them with oxygen (Svetovidov, 1964).

The aim of the present study was to investigate the response of antioxidant enzymes in blenny *Parablennius sanguinolentus* embryos (stage V) (Dekhnik, 1973) exposed to mazut in concentrations of 0.00001, 0.02, 0.1 and 0.2 ml/l.

Materials and methods

Fish egg collection

Eggs were obtained in field conditions at the coastal line of the city of Sevastopol (Black Sea, Russia) during the spawning period in May–June. After collection, the eggs were immediately placed in an aerated tank and transferred to the laboratory. Stage V embryos are characterized by growth of the tail, separation of the tail from the egg yolk, and formation of the heart, gut, liver and fins (Dekhnik, 1973). The eggs were incubated at 12.5–13 °C at different concentrations of mazut.

Preparation of mazut solutions

Mazut (black mineral oil) is a heavy, low-quality fuel oil used in generating plants and similar applications. It may be used as an energy fuel for heating houses and for ships. For experimental purposes, in our study, mazut was mixed at the concentrations of 0.00001, 0.02, 0.1, 0.2, ml/l in filtrated marine water (Black Sea, salinity 18 g/l), and the mixtures were stripped using a magnetic stirrer for 20–30 min; after 30 min the mixture was used in experiments (Chesalina et al., 2000).

Experimental design

Test emulsions of mazut with the concentrations of 0 (control), 0.00001, 0.02, 0.1 and 0.2 ml/l were made using filtrated marine water. Fifty embryos in stage V were randomly transferred into test solutions in 1.5-liter aerated tanks. Each treatment was replicated three times. At the end of the tested developmental stage (72–98 h for stage V) the living embryos were homogenized in cold 0.85 % NaCl using a glass homogenizer, and the homogenates were centrifuged at 8000g for 15 min at cool temperatures. The supernatants were used for biochemical determinations.

Biochemical assays

Antioxidant enzyme activities in the supernatants were determined according to the methods described previously (Rudneva, 2019). The activity of superoxide dismutase (SOD) was assayed on the basis of inhibition of the reduction of nitroblue tetrazolium (NBT) spectrophotometrically at 560 nm. Enzyme activities were calculated as arbitrary units U per (min mg protein)$^{-1}$. Catalase (CAT) activity was measured by the method involving the reaction of hydroperoxide reduction. Enzyme activities were calculated in mg H_2O_2 per (min mg protein)$^{-1}$. Peroxidase (PER) activity was detected by a spectrophotometric method using benzidine reagent. Enzyme activities were calculated in optical units U per (min mg protein)$^{-1}$. Glutathione reductase (GR) activity was assayed spectrophotometrically.

Statistical analysis

Biochemical measurements were detected in duplicate for each sample. Simple, descriptive statistics were performed using ANOVA (Halafian, 2008). A p-value of < 0.05 was used for the determination of statistical significance between control values and the values of the individual experimental group. Statistical correlations between studied biochemical parameters and mazut concentrations in tested experimental groups were calculated by the least-squares method using the computer program CURFVIT (Version 2.10-L).

Results

The activity of studied antioxidant enzymes in developing fish embryos exposed to different concentrations of mazut fluctuated non-uniformly (Fig. 1).

SOD activity varied insignificantly at all tested concentrations with the exception of the concentration of 0.1 ml/l, when enzyme activity increased 3-fold as compared with the control ($p < 0.05$, Fig. 1). CAT activity was significantly elevated at the concentrations of 0.02 and 0.1 ml/l, then it decreased, but it remained higher than the control. The range of PER activity was unclear: it dropped at the concentration of 0.00001 ml/l, at the concentration of 0.002 ml/l it elevated and at mazut level of 0.1 and 0.2 ml/l it decreased again as compared with the control. At all tested mazut concentrations, GR activity in the embryos was significantly lower ($p < 0.05$) as compared with the intact eggs.

The dynamics of enzyme activities in embryos exposed to different mazut concentrations related to the control is presented in Fig. 2.

At all tested mazut concentrations, CAT and PER activity in the fish embryos was higher as compared with the intact eggs. However, SOD activity was significantly greater ($p < 0.05$) at the mazut concentration of 0.1 ml/l and in the case of CAT activity the differences were significant at the mazut concentrations of 0.02 and 0.1 ml/l.

In contrast, PER and GR activities were lower than in the control at all tested mazut concentrations (with the exception of the PER level at toxicant concentration of 0.02 ml/l). PER activity was significantly lower ($p < 0.05$) at the mazut concentration of 0.00001 ml/l and 0.1 ml/l, while the GR level was significantly lower related to the control in all tested mazut concentrations. No correlations were observed between PER and GR activities and mazut concentration. Additionally, the examined enzymes fluctuated independently, and we did not find any correlations between their activities.

Therefore, the response of antioxidant enzymes of fish embryos treated by different concentrations of mazut was non-uniform and depended on toxicant level and enzyme specificity.

Discussion

In the present study, we used as the test organism the embryos of the benthic fish blenny *Parablennius sanguinolentus*, which is distributed in the coastal waters of the Black Sea and could be used as a good model for the oil toxicity effect evaluation both in natural and laboratory conditions. Oil can affect the ecosystem both through its physical influence on the organisms and through the effect of the various chemicals in the oil, which can increase mortality for eggs and larvae, damage the hatching process, and cause development abnormalities (Martin et al. 2014; Karam et al., 2019). Oil can change the physical and chemical properties of marine water (Marimuthua et al., 2019) and stimulate the formation of marine snow (Passow et al., 2012) that may subsequently sink to the marine floor, where it impacts benthic fauna, including demersal fish eggs. In this case the conditions become anoxic, inducing multiplicative stress in marine organisms, which is accompanied by the generation of ROS (reactive oxygen species) (Van der Oost et al., 2003; Lesser, 2006; Sarkar et al., 2006; Cao et al., 2010; Rudneva, 2014; Mu et al., 2018; Beiraoa et al., 2019).

The obtained results demonstrated damage to antioxidant defense and imbalance between oxidative and antioxidant processes in the embryos impacted by mazut. Significantly increased SOD activity was observed in the eggs of blenny, which is due to the production of superoxide radicals generated by mazut as a compensatory embryo response to convert it into H_2O_2 and O_2. CAT is as heme-containing enzyme that facilitates the removal of H_2O_2 by metabolizing it into H_2O and O_2. The present study revealed increased CAT activity together with SOD activity. Therefore, we could note the adaptive response of the eggs to mazut toxicity. A similar study (Rudneva, 2014) showed increased SOD and CAT activities in blenny *Lypophys pavo* exposed to mazut concentration of 0.001, 0.05 and 0.1 ml/l and in the embryos of *Proterorhinus marmoratus* (Rudneva, 2019) exposed to mazut concentration of 0.1 and 1 ml/l. However, the response of SOD and CAT enzymes to the oil impact is not uniform and it depends on the kind of oil, its concentration and the species of fish eggs (Zhang et al. 2004; Paula et al., 2011).

In this case we noted the decrease of PER and GR activities in the impacted blenny embryos. As we noted previously (Rudneva, 2014), PER also plays a role in the processes of eggshell function, because one of its component ovoperoxidase was detected in fish eggs and it uses H_2O_2 as substrate which is generated in the NADPH oxidase system on the surface of eggshell. The disbalance of the antioxidant defense system caused by mazut toxicity inhibited PER activity in impacted blenny. As for glutathione reductase, it is common knowledge that

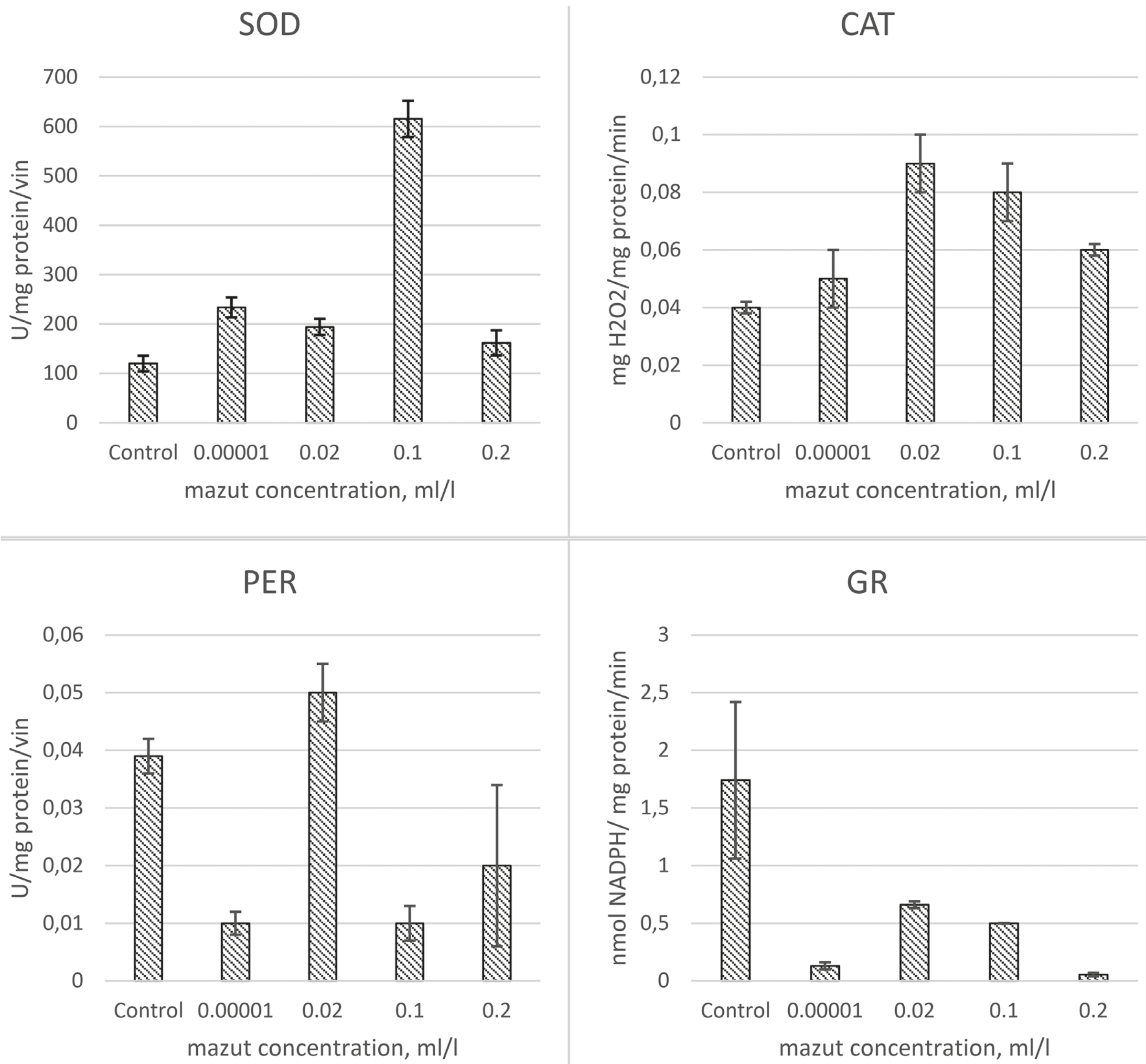

Fig. 1. Antioxidant enzyme activities in developing fish embryos *Blennius sanguinolentus* (stage V) exposed to different concentrations of mazut. SOD — superoxide dismutase, CAT — catalase, PER — peroxidase, GR — glutathione reductase. Enzyme activity is represented as (min mg protein)$^{-1}$, mean ± SD.

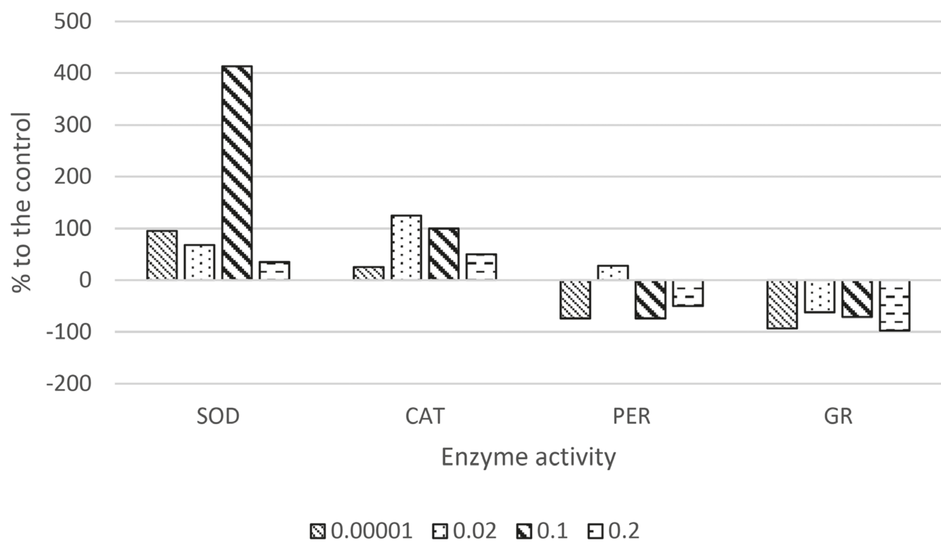

Fig. 2. Dynamics of antioxidant enzyme activities in fish eggs exposed to different mazut concentrations related to the control. The results are presented as percentage of similarity to the control. See Fig. 1 for other definitions.

glutathione is one of the most important antioxidants involved in the protection of the cell membrane from lipid peroxidation by scavenging oxygen radicals and as a substrate of glutathione peroxidase (GPX) and glutathione-S-transferase (GST), which play a role in xenobiotic detoxification. It possesses antioxidant properties and its protective role against toxicity induced by oxidative stress is well known.

Therefore, the obtained results indicate that even organisms in early development stages could stimulate their antioxidant system shortly after exposure to resist the damage of ROS. An increased concentration of oil and its derivates in bottom sediments and in marine water of fish spawning areas causes oxidative stress in their early life stages and induces the imbalance of the oxidant–antioxidant system. Ecotoxicological studies of oil toxicity both in laboratory and field conditions using fish eggs as a test organisms suggest that this mode of action should be considered in the assessment of oil pollution, and indicate the need for a broader approach to understand the aquatic toxicity of different oils.

Conclusions

The present study provides additional evidence for the usefulness of fish egg biochemical biomarkers in assessing the health of aquatic environments. Biomarker variables in the blenny embryos seem to be indicators of oil toxicity in the coastal marine areas and can be used for the development of fish embryo tests for the assessment of water quality. The response of tested antioxidant enzymes was not uniform and depended on mazut concentration. Because of this, measuring a battery of bioindicators can provide insight only into the fish health and ecological status of their habitats. However, links between antioxidant response and contaminants are not definitive and further studies are needed.

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